Rapamycin promotes arterial thrombosis in vivo: implications for everolimus and zotarolimus eluting stents

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Abstract: AIMS: Drug-eluting stents (DES) may be associated with an increased risk for stent thrombosis when compared with bare-metal stents. In endothelial cells, rapamycin induces tissue factor (TF) by inhibiting the mammalian target of rapamycin (mTOR). However, the effect of mTOR inhibition on TF activity and thrombus formation in vivo has not yet been studied. Moreover, it is unclear whether second-generation DES substances everolimus and zotarolimus have an effect on endothelial TF expression. METHODS AND RESULTS: In a mouse carotid artery photochemical injury model, rapamycin (182 +/- 27.5 microg/L) decreased time to thrombotic occlusion by 40%, increased TF activity, and abrogated p70S6K phosphorylation when compared with controls. In vitro, rapamycin, everolimus, and zotarolimus (each 10(-7) mol/l) enhanced TNF-alpha-induced TF expression by 2.2-, 1.7-, and 2.4-fold, respectively, which was paralleled by an increase in TF surface activity. Similar to rapamycin, everolimus and zotarolimus abrogated TNF-alpha-induced p70S6K phosphorylation under these conditions. CONCLUSION: Rapamycin increases TF activity and promotes arterial thrombosis in vivo at concentrations relevant in patients undergoing DES implantation; this effect may increase the thrombogenicity of DES. Since everolimus and zotarolimus augment endothelial TF expression and activity in vitro in a similar manner as rapamycin, these findings may also be relevant for second generation DES.

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Rapamycin promotes arterial thrombosis *in vivo*: implications for everolimus and zotarolimus eluting stents

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Aims Drug-eluting stents (DES) may be associated with an increased risk for stent thrombosis when compared with bare-metal stents. In endothelial cells, rapamycin induces tissue factor (TF) by inhibiting the mammalian target of rapamycin (mTOR). However, the effect of mTOR inhibition on TF activity and thrombus formation *in vivo* has not yet been studied. Moreover, it is unclear whether second-generation DES substances everolimus and zotarolimus have an effect on endothelial TF expression.

Methods and results In a mouse carotid artery photochemical injury model, rapamycin (182 $\pm$ 27.5 μg/L) decreased time to thrombotic occlusion by 40%, increased TF activity, and abrogated p70S6K phosphorylation when compared with controls. *In vitro*, rapamycin, everolimus, and zotarolimus (each 10^{-7} mol/l) enhanced TNF-α-induced TF expression by 2.2-, 1.7-, and 2.4-fold, respectively, which was paralleled by an increase in TF surface activity. Similar to rapamycin, everolimus and zotarolimus abrogated TNF-α-induced p70S6K phosphorylation under these conditions.

Conclusion Rapamycin increases TF activity and promotes arterial thrombosis *in vivo* at concentrations relevant in patients undergoing DES implantation; this effect may increase the thrombogenicity of DES. Since everolimus and zotarolimus augment endothelial TF expression and activity *in vitro* in a similar manner as rapamycin, these findings may also be relevant for second generation DES.

Keywords Drug-eluting stents • Thrombosis • Tissue factor

Introduction

Percutaneous coronary intervention and stenting of the culprit lesion is the preferred treatment in acute myocardial infarction.[1–3] Drug-eluting stents (DES) are associated with reduced restenosis rates when compared with bare-metal stents (BMS).[4–6] However, stent thrombosis rates are not reduced in DES and may even be higher when compared with BMS, particularly in acute coronary syndromes (ACS).[6–13] Since it is associated with a high mortality, stent thrombosis remains one of the most dreaded complications of interventional cardiology. Indeed, stent thrombosis is believed to be one possible reason for the lack of a true mortality benefit with DES when compared with BMS.[14,15] Second-generation DES such as everolimus- and zotarolimus-eluting stents are proposed to display a better safety profile compared with first-generation DES, but long-term, large-scale clinical data supporting this hypothesis are lacking.

Initiation of coagulation is a pivotal event in the pathogenesis of thrombosis and acute myocardial infarction. Tissue factor (TF) is a key trigger of the coagulation cascade; it activates factor X (FX) by binding activated factor VII (FVIIa), which ultimately leads to thrombin formation.[16] Rapamycin, a macrocyclic lacton used on

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first-generation DES, induces TF in human endothelial cells by inhibiting the activity of the mammalian target of rapamycin (mTOR).[17] However, the effect of mTOR inhibition on TF activity and thrombus formation *in vivo* has not yet been studied. Moreover, whether and to what degree the rapamycin analogues everolimus and zotarolimus exert similar effects on endothelial TF expression is unknown. Thus, the present study was designed to investigate the role of mTOR inhibition on TF induction and arterial thrombosis *in vivo* and to examine the effect of everolimus and zotarolimus on TF expression human endothelial cells.

Methods

Carotid artery thrombosis model

C57BL/6 mice (6–8 weeks old; Charles River Laboratories, Sulzfeld, Germany) weighing an average of 23 ± 2 g were treated with rapamycin (2 mg/kg body weight) or with vehicle by intraperitoneal injection. After 1 h, mice were anesthetized with intraperitoneal injection of 2 mg of sodium pentobarbital (Butler, Columbus, OH, USA) as described previously.[18,19] Rose Bengal (Fisher Scientific, Fair Lawn, NJ, USA) was diluted to 10 mg/mL in phosphate-buffered saline (PBS) and then injected into the tail vein in a volume of 0.12 mL at a final concentration of 50 mg/kg with a 27-gauge Precision Glide needle with a 1 mL latex-free syringe (Becton Dickinson, Franklin Lakes, NJ, USA). Once anesthetized, mice were secured in a supine position, placed under a dissecting microscope (Olympus C-4040 Zoom; spatial resolution 4.1 megapixels, Olympus Schweiz, AG, Switzerland), and the right common carotid artery was exposed after a midline cervical incision. A Doppler flow probe (model 0.5 VB, Transonic Systems, Ithaca, NY, USA) was applied to the right common carotid artery and connected to a flowmeter (Transonic, model T106) thus enabling measurement of systolic and diastolic blood flow. Six minutes after Rose Bengal injection, a 1.5 mW green laser light (540 nm; Melles Griot, Carlsbad, CA, USA) was aimed at the right carotid artery and kept at a distance of 6 cm for 60 min or until thrombosis occurred. Exposure of the circulating Rose Bengal to the green laser light triggered a photochemical reaction causing a focal injury mainly confined to the endothelium. From the onset of injury, blood flow in the vessel was monitored for 120 min, at which time the experiment was terminated. Occlusion was defined as a flow of ≤0.1 mL/min for at least 1 min.[19,20] At the time of cessation of blood flow, the appearance of an occlusive thrombus was clearly visible through the microscope in the lumen of the artery.

Tissue factor activity *in vitro* and *in vivo*

Tissue factor cell surface and total tissue activity were analysed in human aortic endothelial cells (HAEC) and mouse carotid artery homogenates, respectively, with a colorimetric assay (American Diagnostica) as described.[19,21]

HAEC were grown in 12-well plates; after stimulation, cells were washed twice with PBS followed by incubation with human FVIIa and FX at 37°C, resulting in the formation of TF/FVIIa complex at the cell surface. Right carotid arteries were homogenized in 50 μL of lysis buffer (50 mmol Tris–HCl, 100 mmol NaCl, 0.1% Triton X-100, pH 7.4) 1 h after intraperitoneal application of rapamycin (2 mg/kg body weight) or vehicle and left to stand on ice for 30 min. In either case, the TF/FVIIa complex converted human FX to factor Xa, which was measured by its ability to cleave a chromogenic substrate. A standard curve with lipidated human TF was performed to ensure that measurements were taken in the linear range of detection (data not shown).

Cell culture

HAEC were cultured as described.[21] Cells were grown to confluence in 3.5 cm dishes and rendered quiescent for 24 h before stimulation with TNF-α (Sigma, Munich, Germany). Rapamycin, everolimus (both from Sigma), and zotarolimus (a kind gift from Abbott Vascular, Santa Clara, CA, USA) were added 60 min prior to stimulation.

Western blot analysis

Protein expression was determined by western blot analysis.[17] Cells were lysed in 50 mM Tris buffer and 30–40 μg samples were loaded and separated by 10% SDS–PAGE. Proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, USA) by semidry transfer. The antibody to human TF (American Diagnostica, Pfungstadt, Germany) was used at 1:1000 dilution; antibodies against the phosphorylated Thr-389-residue of p70S6 kinase (S6K) and against total S6K (both from Cell Signaling) were used at 1:1000 and 1:2000 dilution, respectively. Blots were normalized to GAPDH expression (1:20 000 dilution, Sigma).

One hour after intraperitoneal application of rapamycin (2 mg/kg body weight) or vehicle (2.5% DMSO in PBS), aortas of mice were homogenized in 50 μL of lysis buffer (50 mmol Tris–HCl, 100 mmol NaCl, 0.1% Triton X-100, pH 7.4) and left to stand on ice for 30 min. One hundred microgram samples were loaded and separated by 10% SDS–PAGE; proteins were transferred to a PVDF membrane and probed with an antibody to phosphorylated p70S6K (1:1000 dilution) or total p70S6K (1:2000 dilution).

Statistics

Data are presented as mean ± SD. For the comparison of two groups, unpaired Student's *t*-test and Mann–Whitney test were applied for normally and non-normally distributed variables, respectively. ANOVA with Bonferroni's correction was used for comparison of greater than or equal to three groups (all variables in multi-group comparisons were normally distributed). A P-value < 0.05 was considered significant.

Results

Rapamycin inhibits mammalian target of rapamycin, increases tissue factor activity, and promotes arterial thrombosis *in vivo*

Photochemically induced arterial injury, an established model of arterial thrombosis,[18–20,22] was applied to study arterial thrombus formation. Vehicle-treated mice developed thrombotic occlusion within 36.0 ± 15.6 min (*Figure 1A* left column). Treatment with rapamycin (2 mg/kg body weight) shortened time to thrombotic vessel occlusion by 40% to 21.4 ± 6.4 min ($P < 0.05$; *Figure 1A*, right column). Plasma levels of rapamycin 1 h after intraperitoneal injection were 182 μg/L (± 27.5 μg/L; $n = 4$).

Photochemical arterial injury is dependent on TF.[18] Treatment of mice with rapamycin (2 mg/kg body weight) increased TF activity in carotid arteries by 45% when compared with TF activity in carotid arteries of vehicle-treated mice (*Figure 1B*; $P = 0.01$).

Phosphorylation of p70S6K, a downstream target of mTOR, is frequently used to assess mTOR inhibition by rapamycin,[17,23,24] and abrogation of p70S6K phosphorylation leads to an increase in TF expression and activity.[17] While a prominent

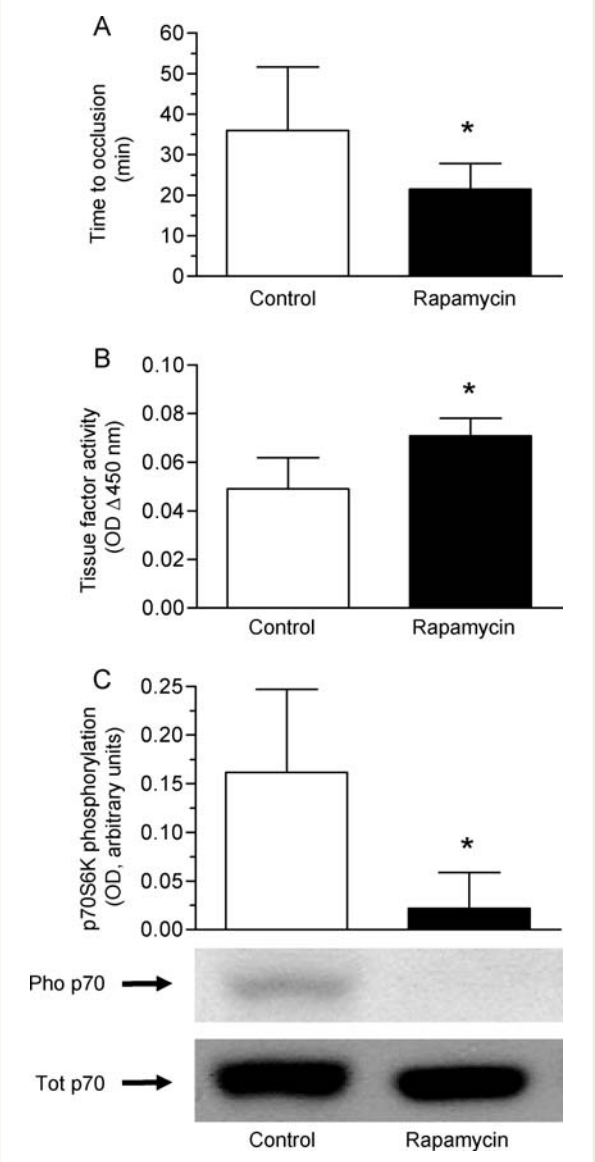

Figure 1 Rapamycin inhibits mammalian target of rapamycin (mTOR), increases TF activity, and promotes arterial thrombosis *in vivo*. (*A*) Treatment with rapamycin shortens time to thrombotic carotid artery occlusion. $^*P < 0.05$ vs. vehicle-treated mice; $n \geq 6$ for each group. (*B*) Rapamycin increases TF activity in mouse carotid artery. Values are given as absorbance at 405 nm. $^*P = 0.01$ vs. vehicle-treated mice, $n = 5$ for each group. (*C*) Treatment with rapamycin inhibits mTOR activity *in vivo* as evidenced by reduced p70S6K phosphorylation. $^*P < 0.01$ vs. vehicle-treated mice, $n = 5$ for each group.

phosphorylation of p70S6K was observed in aortas of mice treated with vehicle, it was abrogated in rapamycin-treated animals (*Figure 1C*; $P < 0.01$).

There was no change in activated partial thromboplastin time (25.5 ± 1.22 vs. 24.4 ± 1.81 s, $P =$ n.s.) or prothrombin time (PT; 11.1 ± 0.2 vs. 11.4 ± 0.43 s, $P =$ n.s.) in rapamycin-treated vs. control mice, respectively.

Rapamycin enhances tissue factor expression in human endothelial cells

Stimulation of HAEC with TNF-α (5 ng/mL) induced TF protein expression (*Figure 2*). Rapamycin (10^{-9}–10^{-7} mol/L) resulted in a concentration-dependent enhancement of TF expression with a maximal increase of 1.9-fold when compared with stimulation with TNF-α alone and 68-fold when compared with the unstimulated control (*Figure 2A*; $P < 0.02$ for rapamycin 10^{-7} mol/L vs. TNF-α alone). Rapamycin (10^{-7} mol/L) abrogated p70S6K phosphorylation (*Figure 2B*).

Everolimus and zotarolimus enhance tissue factor expression in human endothelial cells

Like rapamycin, everolimus (10^{-9}–10^{-7} mol/L) and zotarolimus (10^{-9}–10^{-7} mol/L) enhanced TNF-α-induced TF expression in a concentration-dependent manner ($n = 3$; $P < 0.05$, data not shown). When compared side-by-side (each substance 10^{-7} mol/L), the maximal increase in TF induction was 2.2-, 1.7-, and 2.4-fold for rapamycin, everolimus, and zotarolimus, respectively, when compared with TNF-α (*Figure 3A*; $P < 0.005$ for everolimus vs. TNF-α alone and $P < 0.001$ for zotarolimus vs. TNF-α alone). Tissue factor induction by zotarolimus+TNF-α was 1.4-fold higher when compared with everolimus+TNF-α ($P < 0.05$).

Rapamycin increased TNF-α-induced TF surface activity by 1.5-fold; similarly, everolimus and zotarolimus augmented TNF-α-induced TF surface activity by 1.4-fold and 1.5-fold, respectively (*Figure 3B*; $P < 0.01$ for everolimus or zotarolimus vs. TNF-α alone). Similar to rapamycin (*Figure 2B*), everolimus (*Figure 3C*, upper panel) and zotarolimus (*Figure 3C*, lower panel) abrogated TNF-α-induced p70S6K phosphorylation.

Discussion

Rapamycin was previously shown to induce TF *in vitro*; yet, the physiological significance of this effect has not been assessed.[17] The findings of the present study suggest that this effect is also relevant *in vivo*, since inhibition of mTOR by rapamycin increases TF activity and promotes arterial thrombosis in the mouse carotid artery exposed to laser injury. Moreover, everolimus and zotarolimus inhibit mTOR and enhance TF expression and activity in human endothelial cells similar to rapamycin, indicating that these findings may be relevant both for first- and second-generation DES.

The inhibitory role of the mTOR on TF expression is established, as its inhibition enhances TF expression in response to a variety of mediators *in vitro*.[17,19,25] Binding of rapamycin to its intracellular receptor, FKBP-12, leads to the formation of the rapamycin–FKBP-12 complex, which in turn inhibits mTOR activity. Phosphorylation of the downstream target of mTOR, p70S6K, is widely used as a readout for the inhibitory effect of rapamycin,[23,24] since mTOR-dependent phosphorylation of the Thr-389 residue of p70S6K is necessary for its activity.[23] Rapamycin is known to abrogate p70S6K phosphorylation,[17] and the present study demonstrates that the rapamycin analogues

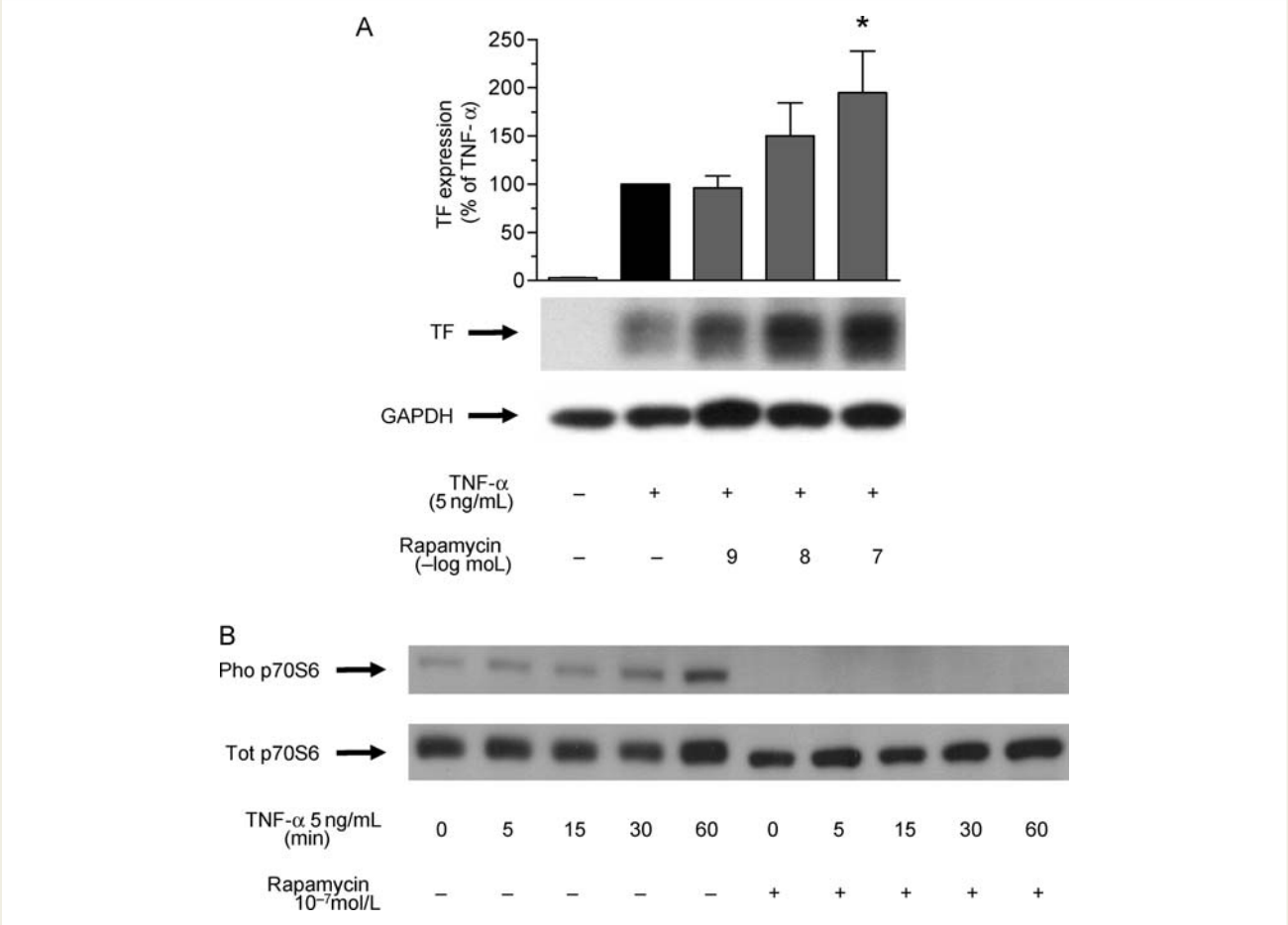

Figure 2 Rapamycin inhibits mammalian target of rapamycin and enhances endothelial tissue factor expression in human endothelial cells. (*A*) Rapamycin enhances TNF-α-induced TF expression in a concentration-dependent manner. *$P < 0.02$ vs. TNF-α alone. (*B*) Rapamycin completely abrogates p70S6K phosphorylation.[17] Total levels of p70S6K remain unchanged. Blots are representative of two independent experiments.

everolimus and zotarolimus equally do so, supporting the interpretation that inhibition of mTOR promotes TF induction.[17]

Biologically active TF (which is located at the cell surface) is induced by all three drugs to a very similar degree *in vitro*. In the case of rapamycin and zotarolimus, the increase in TF surface activity was not as pronounced as that in protein expression. Similar discrepancies between the degree of protein expression and surface activity have also been observed in previous studies,[17,21] and may be accounted for by the expression of encrypted TF and/or the distribution of TF in several cellular compartments.[26]

The dose of rapamycin used in our *in vivo* experiments (2 mg/kg body weight) corresponds to that employed in previous murine studies.[27] Rapamycin plasma levels 1 h after intraperitoneal injection (i.e. at the time of thrombosis) were 182 μg/L, corresponding to 2×10^{-7} mol/L; hence, plasma levels *in vivo* were similar to the concentrations employed *in vitro*. Maximal systemic concentrations of rapamycin after deployment of two sirolimus-eluting stents are reported to be ~1 ng/mL (~1.15×10^{-9} mol/L).[28] Although difficult to assess, local concentrations after stent deployment at the cellular level are likely to be significantly higher than systemic levels due to the lipophilic properties of these agents, which tend to accumulate in the vessel wall.[8,29–31] Hence, the concentrations used in our study both *in vivo* and *in vitro* are likely to be relevant for patients treated with DES.

When employed to prevent organ rejection after transplantation, rapamycin target levels are around 10 ng/mL (corresponding to 10^{-8} mol/L). Interestingly, cases of thrombotic microangiopathy have been described during treatment with rapamycin (with or without a calcineurin inhibitor) in transplant recipients with chronic graft-vs.-host disease as well as after renal transplantation.[32,33] Thrombotic complications were also observed in patients treated with rapamycin after liver transplantation.[33] The concentrations used in our *in vivo* experiments are around 10-fold higher than target levels after transplantation, which would explain why these complications do not occur more frequently. However, it is conceivable that under certain circumstances, rapamycin may have a prothrombotic effect in patients receiving the drug to prevent organ rejection.

In spite of the obvious success of DES, several shortcomings have been noted since their introduction including the risk of stent thrombosis.[12] While some studies found similar thrombosis

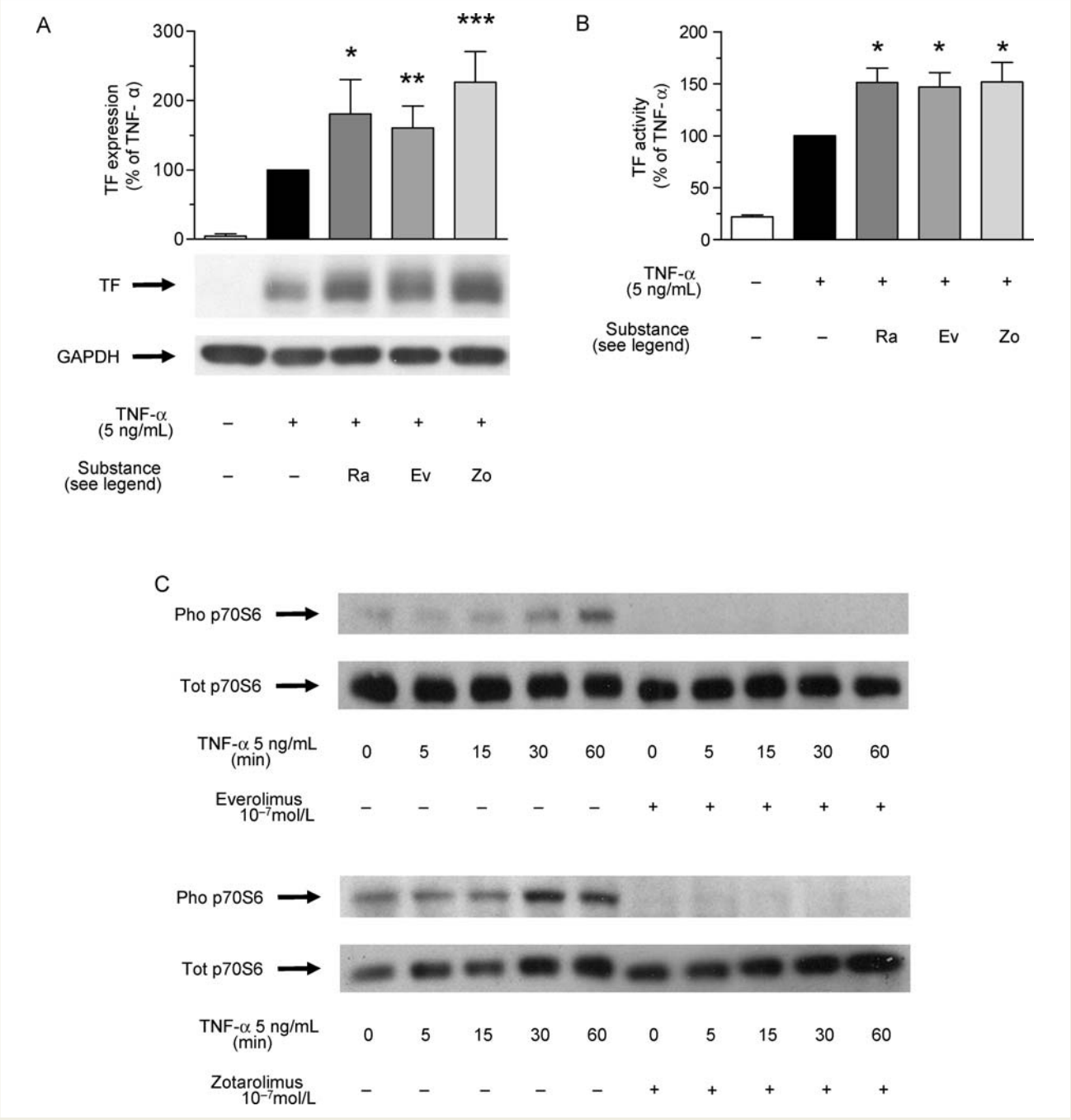

Figure 3 Everolimus and zotarolimus induce tissue factor expression and surface activity. (*A*) Comparison of maximal increase in rapamycin-, everolimus-, and zotarolimus-enhanced TF expression. $*P < 0.02$ and $**P < 0.005$ vs. TNF-α alone; $***P < 0.001$ vs. TNF-α alone and $***P < 0.05$ vs. everolimus (using ANOVA with Bonferroni's correction). Blots are representative of at least three independent experiments, and values are given as percentage of stimulation with TNF-α alone. (*B*) Rapamycin, everolimus, and zotarolimus enhance TNF-α-induced TF surface activity. Values are given as percentage of stimulation with TNF-α alone. $*P < 0.01$ vs. TNF-α alone; $n = 3$. Everolimus (*C*, upper panel) and zotarolimus (C, lower panel) abrogate p70S6K phosphorylation. Total levels of p70S6K remain unchanged. Blots are representative of four independent experiments. TF, tissue factor, Ra, rapamycin, Ev, everolimus, Zo, zotarolimus.

rates in first-generation DES and BMS,[34,35] others have described an increased risk in DES.[7,10–12] Even though long-term large-scale studies are currently underway to assess this issue, the fear of stent thrombosis remains high owing to the oftentimes severe clinical consequences. The occurrence of stent thrombosis has been reported both with everolimus- and zotarolimus-eluting stent systems;[36,37] as the number of patients studied and the follow-up duration are still limited, it is so far impossible to infer, whether

the risk of stent thrombosis is increased in such second-generation DES when compared with first-generation DES or BMS.

Several mechanisms of stent thrombosis are currently discussed, of which delayed endothelial healing is presently thought to be most relevant.[12] Impaired re-endothelialization may be a particularly important cause of stent thrombosis in conditions with local inflammation, such as in ACS. In a recent autopsy study of patients treated with DES for acute myocardial infarction, vessel healing at the culprit site was indeed substantially delayed when compared with those treated for stable angina, suggesting an increased thrombotic risk in the former group of patients.[38] Our data demonstrating enhanced TF activity as well as increased arterial thrombogenicity in the setting of mTOR inhibition by rapamycin support the interpretation that TF may also play an important role in the pathogenesis of stent thrombosis, which may be even more pronounced in the inflammatory microenvironment of acute myocardial infarction. Since the mouse injury model used in this study is mainly confined to the endothelium without exposing arterial wall smooth muscle cells previously shown to contain prothrombin that is rapidly converted to thrombin after injury,[39] this model probably underestimates the thrombotic stimulus seen with usual angioplasty and stenting. Rapamycin-coated stents elute about 80% of the drug by 30 days.[4,5] However, rapamycin easily penetrates cell walls due to its lipophilic properties resulting in long-term retention of the drug in arterial tissue.[29–31] Hence, the time course of re-endothelialization as well as the kinetics of rapamycin release imply that rapamycin-enhanced endothelial TF expression may potentially play a role in the pathogenesis of acute and subacute stent thrombosis, while it may be less relevant for late stent thrombosis. Importantly, PT, which by virtue of its measurement is dependent on FVIIa and exogenous TF, remained unchanged after treatment of mice with rapamycin, indicating that an increase in vessel-wall-derived TF is responsible for the observed enhanced thrombogenicity. To expand on these observations, further studies are needed to investigate the extent and the pattern of TF expression in the arterial wall after deployment of both first- and second-generation DES.

Furthermore, substances eluted from DES may have an effect not only on the stented arterial segment, but also on the endothelium distal to the stent. Indeed, impaired endothelial function was demonstrated by quantitative coronary angiography in coronary arteries distal to rapamycin-eluting stent implantation.[40,41] Thus, rapamycin, everolimus, or zotarolimus may not only increase TF in the stented segment, but also in endothelial cells in the immediate vicinity as well as in the more distal circulation. Such an effect may potentially contribute to no-reflow phenomena after stent deployment.

While the current study compares the *in vitro* TF induction of rapamycin, everolimus, and zotarolimus, it does not compare TF activity and thrombus formation *in vivo* amongst the three drugs; in order to do so correctly and adequately, measurement of *in vivo* plasma levels over time of all three drugs as well as appropriate adjustment of the applied doses of the drugs would be required, which was beyond the scope of the current study. Instead, we demonstrate for the first time *in vivo* (as a proof-of-principle) that rapamycin inhibits arterial mTOR activity, increases arterial TF activity and promotes thrombus formation without affecting systemic coagulation parameters. Given the similar effect of everolimus and zotarolimus on TF expression as well as on p70S6K phosphorylation *in vitro*, an increase in TF activity and promotion of thrombus formation *in vivo* is nevertheless likely to occur with these two drugs as well.

In summary, this study reveals that inhibition of mTOR increases TF activity and promotes arterial thrombosis *in vivo*, which may favour the development of thrombosis in DES. As everolimus and zotarolimus equally increase TF expression and activity *in vitro*, these findings may be relevant for both first- and second-generation DES.

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